Conclusions

Although essential oil analysis is now a well-established field, further work is needed, not only to improve sample preparation and analysis techniques, but also to deal with one of the main aims of this research field: to isolate and elucidate the structure of new odorous compounds. These studies evolve along two main lines. The first and classical one combines isolation and spectroscopic techniques and mainly concerns new mono- and sesquiterpenoids. The second mainly involves the so-called supervolatile fraction and perfumed trace compounds, two fractions that play a fundamental role in odour impact. For the supervolatile fraction, some topics requiring further study are HS combined with effective cryotrapping techniques, systems for direct GC injection of large volumes of gas samples and GC columns with a high retention capacity. For compounds present in the essential oil at the p.p.m. level (e.g. pyridine derivatives in peppermint and orange oils), a number of points would benefit from further investigation. These include increased selectivity of sample preparation techniques and increased sensitivity and selectivity of analysis techniques.

See also: **II/Chromatography: Gas:** Column Technology; Detectors: Mass Spectrometry; Detectors: Selective; Headspace Gas Chromatography; Sampling Systems; **III/Essentials Oils:** Distillation. **Terpenoids: Liquid Chromatography**.

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Thin-Layer (Planar) Chromatography

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Introduction

Essential oils are mixtures of mainly volatile components belonging to different chemical classes. They

are characterized by a pleasant smell and are generally obtained by steam distillation of aromatic plants, with the exception of citrus peel essential oils, which are produced by cold-pressing the peel of the fruits. This process involves the abrasion of peel and the removal of the oil in an aqueous emulsion that is subsequently separated in a centrifuge. Other methods may be water distillation or extraction with sub- or supercritical fluids. Essential oils are not soluble in water, but are quite soluble in alcohol.

Resins can be either natural or prepared: natural resins are exudations from trees or plants, and are formed in nature by the oxidation of terpenes; prepared resins are oleoresins from which the essential oils have been removed. Resins are mixtures of many components; they are solid, amorphous, more or less coloured, nonvolatile, with a characteristic smell, insoluble in water, but soluble in alcohol or other organic solvents.

Balsams are natural raw materials exuded from a tree or a plant; they have a high content of benzoic acid, benzoates or cinnamates.

The main constituents of essential oils, balsams and resins are terpene or aromatic hydrocarbons, and their oxygenated derivatives (alcohols, aldehydes, esters, ketones, oxides, etc.).

The physiological role of oils and resins in plants and trees is not well understood. It is likely that they play a role as lures for insects. They may also serve to protect plants from parasites, increase the rate of transpiration and act as a seal for wounds. They are largely used in perfumery, food or pharmaceutical industries, as flavouring agents or because of their different pharmacological actions.

Characterization of Essential Oils, Balsams and Resins

Essential oils may be characterized by the determination of physicochemical properties such as boiling point, freezing point, solubility, density, optical rotation, refractive index, etc. These parameters can also help in the detection of adulteration. For the study of the qualitative and quantitative composition of essential oils and resins, chromatographic methods are the techniques of choice. Since the main part of the oil consists of volatile components, gas chromatography (GC) equipped with FID (flame ionization) or MS (mass spectrometer) detectors is the most used technique.

High performance liquid chromatography (HPLC) is also widely used for separation of semi-volatile or nonvolatile components, for preparative purposes, or for the analysis of thermally labile components. The limitation of HPLC is detection, because many components of essential oils do not absorb in the UV-visible region, and UV detectors are the most popular in HPLC. Thin-layer chromatography (TLC) is a very widely used chromatographic technique, and modern HPTLC can be advantageously used instead of HPLC or GC in many analytical situations.

Some of the advantages of TLC are its simplicity, economy in materials, simultaneous analysis of a large number of samples and the use of complementary post-chromatographic universal and selective detection methods. Some disadvantages of TLC, such as the long times required for development or the difficulties in controlling the speed of solvent migration, may be overcome by OPLC (overpressured layer chromatography). OPLC, developed by Tyihak and co-workers at the end of the 1970s, is a planar liquid chromatographic technique with advantages over classical TLC and HPLC: the stationary phase is covered completely by a flexible membrane under external pressure and, because the eluent is introduced to the layer by means of a pump, the solvent flow rate may be controlled. Improved separation efficiency, shorter time requirement, better resolution, and lower solvent consumption than classical TLC or HPLC can thus be obtained. Moreover, OPLC can be used for analytical or preparative purposes and maintains all the advantages already mentioned for classical TLC.

Another advance in the development of TLC is chromatography on permanent rod-shaped layers (Chromarods), whose mechanical and chemical properties permit detection of the separated components in an ionization detector; this technique was developed in the late 1960s and can be successfully used for quantitative separations of substances that cannot be analysed by GC because of their low volatility.

There are many papers on planar chromatography that well illustrate the most recent developments of this technique: the use of multidimensional development, the coupling with particular detectors such as MS or FT-IR detectors, or the use of fully computerized image processing instruments. All these developments are accompanied by improvements in the performance and in the reproducibility of precoated layers for planar chromatography.

The literature reports some applications of planar chromatography to the analysis of essential oils, balsams and resins, to obtain different information. Methods have been developed that use both conventional TLC, HPTLC and OPLC for analytical and/or preparative separations. Only a few papers have been published on the separation of terpenes and related substances on Chromarods. One of the reasons for this is probably the volatility of terpenes of low

relative molecules mass, leading to low, irreproducible FID response. Resins have been separated from arnica (benzene/chloroform, 67 : 33) and Tolu balsam (benzene/chloroform/formic acid, 69 : 29 : 2), but the results obtained were not satisfactory. The number of applications is limited, if compared with the applications of GC or HPLC to the analysis of essential oils and plant extracts. However, often TLC is essential as a simple analytical and preparative technique. A recent review summarized the chromatographic methods reported by the *European Pharmacopoeia* (2nd edn) for the analysis of products from medicinal plants, including essential oils, balsams and resins. Over half of the methods reported for medicinal plant products are chromatographic methods, of which TLC represents 82%.

Preparative TLC

Essential oils and resins are complex mixtures containing numerous components that belong to different chemical classes present in different concentration. Often it is necessary to fractionate the mixture into single chemical classes or to isolate single components, particularly useful for the determination of properties such as authenticity, geographical origin or pharmacological activity.

This fractionation can be carried out by preparative TLC. For example, for detecting illegal adulteration of pharmacognostic mint (*Mentha arvensis*) or peppermint (*M. piperita*) oils with racemic menthyl acetate, samples were separated by TLC (polygram SIL G/UV plates, CH_2Cl_2 as mobile phase) and the zone of menthyl acetate extracted from the plate and analysed by chiral capillary GC. Since natural mint oils contain 100% of the (−)-isomer of menthyl acetate, the presence of the (+)-isomer can be used to quantify this kind of adulteration. In this case, preseparation is necessary to obtain a reliable stereodifferentiation without problems of peak overlap in a direct GC analysis.

TLC has been used as preparative tool to obtain pure components to be used for further characterization. For example, components of the sesquiterpene fraction of camomile oil were separated by semipreparative TLC. The components so obtained – *trans*-farnesene, chamazulene, *cis*-en-in-dicycloether, *trans*-en-in-dicycloether, (−)-α-bisabolol, (−)-α-bisabolol oxide A, and (−)-α-bisabolol oxide B – were analysed by GC-MS, and the MS spectra were used to build a database since mass spectra of these components are not included in some commercial MS libraries. **Figure 1** shows the TLC separation obtained using silica gel TLC plates. **Table 1** reports the R_F values and identification of components.

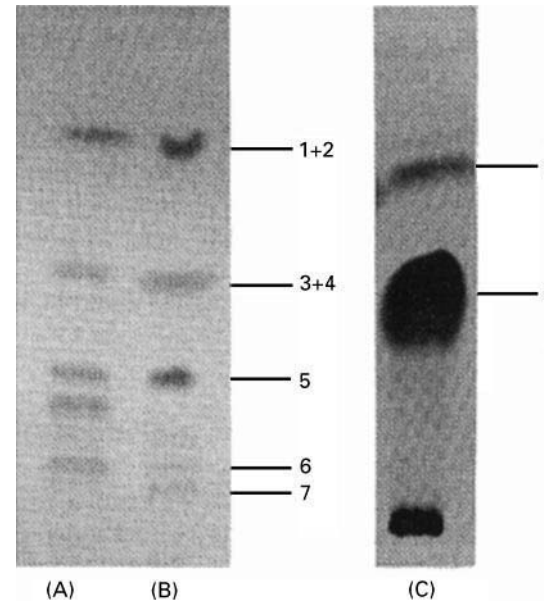

Figure 1 TLC chromatograms of tetraploid camomile (CFH) essential oil obtained by steam distillation (A), compounds isolated from CFH oil (B) and separation of *trans*-farnesene and chamazulene (C). Mobile phase: (A) and (B) dichloromethane/toluene/ethyl acetate (70 : 28 : 2, v/v/v); (C) cyclohexane. Detection by spraying the plates with 1% vanillin solution. (Reproduced with permission from Zekovic Z, Pekii B, Lepojevic Z and Petrovic L (1994) *Chromatographia*, 39: 587–590.)

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Preparative separations have also been carried out by OPLC to isolate antifungal compounds of the essential oil obtained by water distillation of the fresh bark of *Ocotea usambarensis* from Rwanda. The strategy followed for characterization of essential oil constituents is illustrated in **Figure 2**. This scheme shows how a combination of chromatographic techniques, including TLC, can be useful to characterize bioactive constituents of medicinal plants.

Quantitative Analysis by TLC

Some methods have been developed for the quantitative analysis of the main volatile components of essential oils by TLC as a rapid and easy alternative to other chromatographic determinations, in particular GC and HPLC which are more expensive and time-consuming.

It is difficult to find applications of TLC densitometry to problems in the field of essential oils, e.g. for the quantification of individual components, probably because of the volatility of the various components. One example is the quantitative determination of linalool, linalyl acetate and terpinen-4-ol in lavender oil. The first two compounds are the main components of the oil.

Table 1 hR_F values of camomile oil components separated in Figure 1

N	*Compounds*	*hR*$_F$ (%)		*Colour*
		A, B	*C*	
1	*Trans*-farnesene	93.22	64.61	Dark green
2	Chamazulene		46.15	Dark red
3	*Cis*-en-in-dicycloether	63.13	–	Light brown
4	*Trans*-en-in-dicycloether		–	
5	(−)-α-Bisabolol	41.53	–	Violet
6	(−)-α-Bisabolol oxide A	20.76	–	Yellow
7	(−)-α-Bisabolol oxide B	16.94	–	Yellow

A,B, mobile phase dichloromethane/toluol/ethyl acetate (70 : 28 : 2, v/v/v). C, mobile phase cyclohexane. (Reproduced with permission from (1994) *Chromatographia*, 39: 587–590.)

Figure 3 shows the TLC and GC analysis of a lavender oil. The quantitative results obtained with the two techniques are comparable. This result shows that TLC densitometry is a good technique for both qualitative and quantitative analysis of the main components of essential oils. It can be useful in the identification of an oil, and can simultaneously also detect less volatile components.

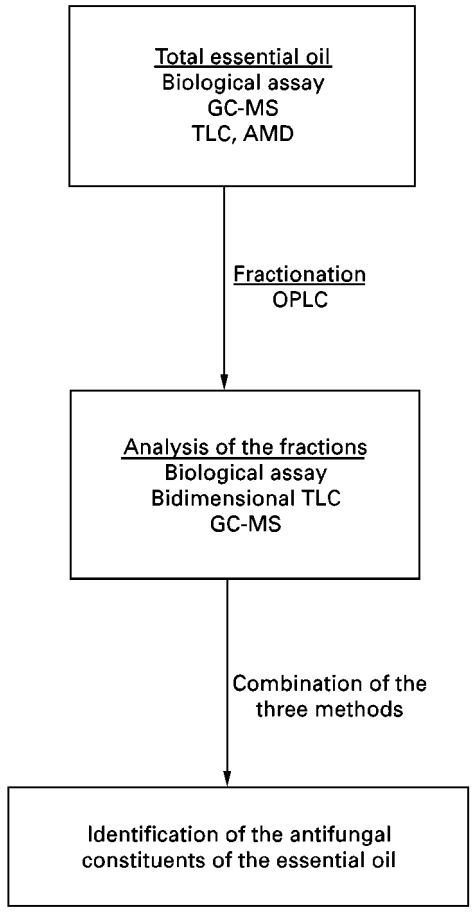

Figure 2 Strategy for the characterization of essential oil constituents from the bark of *Ocotea usambarensis* (Laureaceae). (Reproduced with permission from Hostettmann K., Terreaux, C, Marston, A and Petterat O, (1997) *Journal of Planar Chromatography*, 10: 251–257.

Other examples are the quantitative determination of citral in citrus oils and 1,8-cineole in eucalyptus oils. The determination of citral (a mixture of two terpene aldehydes, neral and geranial) is of particular importance for citrus oils, mainly for lemon oils. TLC determination of citral has been carried out on silica gel plates, developed with hexane/chloroform (70 : 30) and measured at 250 nm with a TLC scanner. The results were compared with those obtained by GC, and the ratio between TLC and GC values was constant at 0.8.

Eucalyptus essential oil contains a high amount of 1,8-cineole, an oxygenated monoterpene. In recent years, interest in developing new uses for cineole-rich eucalyptus oils has been renewed. The determination of 1,8-cineole by TLC has been carried out using silica gel plates and a mixture of light petroleum/chloroform (70 : 30) as mobile phase. Visualization is with 4-dimethylaminobenzaldehyde-S reagent (4-DMAB) and quantitation is with a scanner. Quantitative results compared with GC-MS data were found to be essentially identical.

TLC for Detection of Authenticity and Botanical Origin

TLC can be used for screening samples to establish their authenticity and botanical origin. An example is the determination of the quality of cinnamon by TLC densitometry. Cinnamon is one of the oldest known spices; the cinnamons of commerce are derived from the dried inner bark of several species of *Cinnamomum*. Cinnamon oil derived from the inner bark of *Cinnamomum zeylanicum* Nees is generally

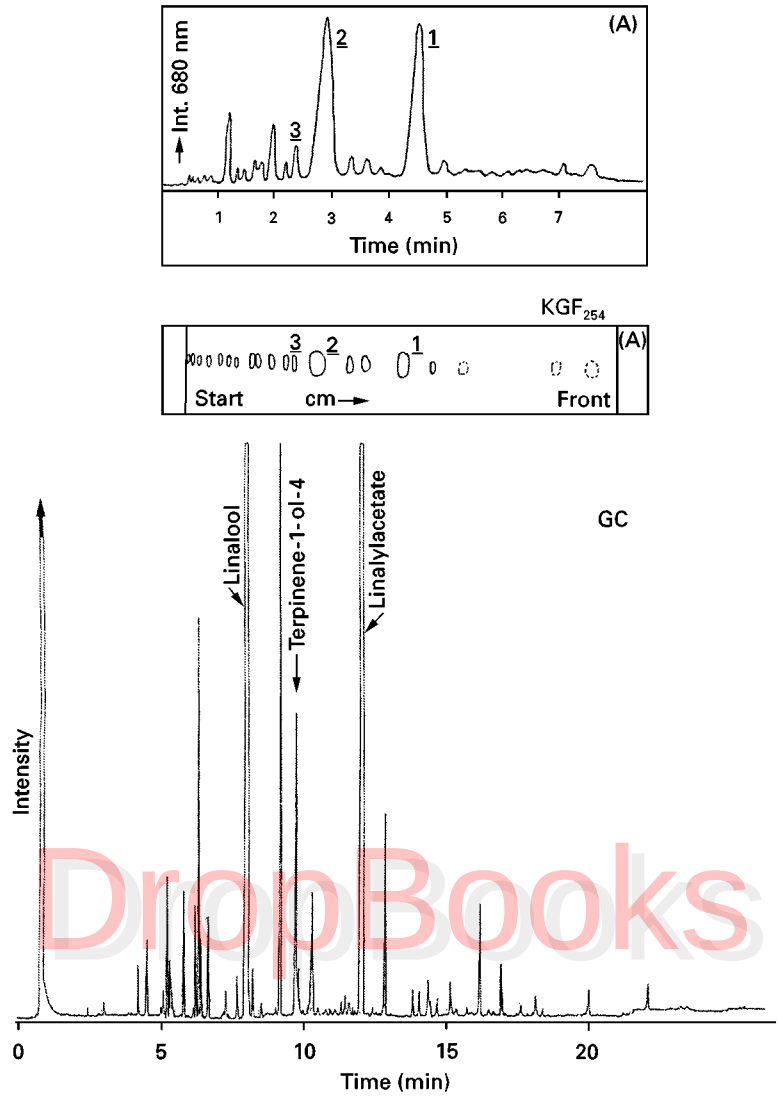

Figure 3 TLC (middle), densitometry (upper) and GC (lower) of lavender oil. TLC conditions: 20 × 20 cm plates, coated with 0.25 mm silica gel 60. Mobile phase: dichloromethane/methanol, 10 : 1.

Detection: anysaldehyde/sulfuric acid ($\lambda = 680$ nm); copper-acetate/phosphoric acid (white light); copper-sulfate/phosphoric acid followed by sulfuric acid spray (white light).

Sample volume: 15 mL of a 5% dichloromethane solution of oil or pure standard components. GC conditions: 25 m × 0.32 mm i.d. HP-5 fused silica capillary column (0.17 μm film thickness); carrier gas: H_2; detector (FID) and injector (split) at 250°C. Temperature program: 60–240°C, 6°C min^{-1}. (Reproduced from (1989) *Mikrochim. Acta (Wien)*, 3: 1–6, with permission from Springer-Verlag, Wien.)

considered to have a better flavour, and commands the highest price.

This oil can be adulterated with cinnamon leaf or root bark oils, that have a different composition from inner bark oil and are less valuable. Moreover, some countries consider oil obtained from *C. cassia* Blume as cassia oil, and it is unlawful to present cassia as cinnamon, or to prepare mixtures of the two and present the result as cinnamon.

Figure 4 shows the TLC densitometry separation of polar aromatic semivolatile compounds of cinnamon and cassia. As can be seen, cinnamon and cassia oils can be easily distinguished because of the presence of higher amount of coumarin and the absence of eugenol in cassia oil.

Another example is the method developed to discover the poisonous Japanese star anise or shikimi fruits (*Illicium anisatum*), when they are mixed with those of the Chinese anise or star anise (*I. verum*). Miristicin only occurs in the essential oil of shikimi, albeit in small amounts, but it is absent in star anise. TLC allows the detection of an admixture of only 5% shikimi, as shown in **Figure 5**.

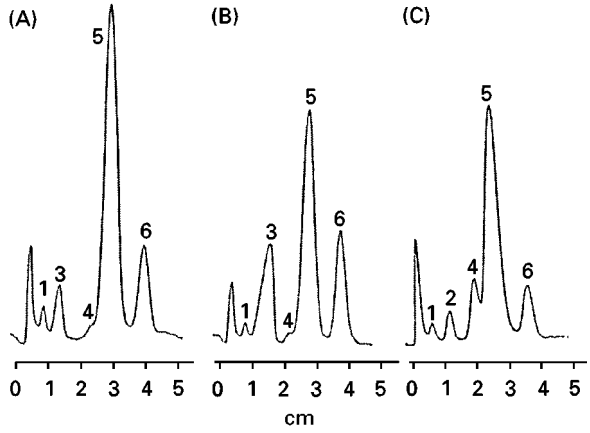

Figure 4 Separation of cinnamon and cassia oils by silica gel HPTLC plates. (A) Authentic sample of essential oil from the inner bark of *Cinnamomum zeylanicum* Nees; (B) cinnamon oil adulterated with leaf oil; (C) authentic sample of cassia oil from *C. cassia* Blume. Peaks: 1, cinnamyl alcohol; 2, coumarin; 3, eugenol; 4, 2-methoxy-cinnamaldehyde; 5, *trans*-cinnamaldehyde; 6, cinnamyl acetate. TLC conditions: TLC plates predeveloped in hexane/triethylamine (46 + 4, v/v) and separation in unsaturated chamber with hexane/chloroform/triethylamine (90 + 6 + 4, v/v/v). The separation was scanned at $\lambda = 255$ nm. (Reproduced with permission from Poole SK, Kiridena W, Miller KG and Poole CF (1995) *Journal of Planar Chromatography*, 8: 257–268.

Determination of the Antioxidant Activity of Essential Oils

The versatility of TLC is also illustrated in the following example, where the composition and the antioxidant activity of essential oils from oregano plants grown wild in Greece is studied. The composition has been studied by GC-MS (ITD) and carvacrol found to be the main components. A variety of oregano, *O. vulgare* subsp. *hirtum*, has a high thymol content, while the other plant species contain less than 1% of thymol. The antioxidant activity of essential oils, pure carvacrol, thymol and butylated hydroxytoluene (BHT) was tested on silica gel TLC plates. The plates were sprayed with a solution of β-carotene and linoleic acid and then exposed to daylight until the background colour disappeared. Spots of components having antioxidant activity remained yellow for a longer period.

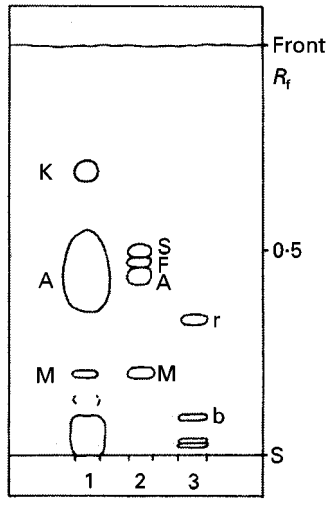

Figure 5 TLC chromatogram of (1) mixture of shikimi fruit and star anise fruit oils; (2) mixture of standard compounds; and (3) reference mixture. Key: A, anethole; F, foeniculin; M, myristicin; S, safrol; r, red; b, blue. (Reproduced from (1990) *Deutsche Apotheker Zeitung*, 130: 1194–1201, with permission from Deutscher Apotheker, Verlag.)

TLC for the Analysis of Phenolic Compounds

TLC has been used to analyse less volatile components such as flavonoids, phenolic acids or coumarins. These classes of components are very important for the characterization of plant materials, and can have specific pharmacological activities. For example, the spasmolitic activity of camomile is mainly due to the presence of flavonoids apigenin, apigenin-7-*O*-β-glucoside and its acetylated derivatives. **Figure 6** show the HPTLC chromatogram of camomile flavonoids. HPTLC is the fastest chromatographic method for qualitative identification of apigenin and its glucosides in camomile. Quantitative

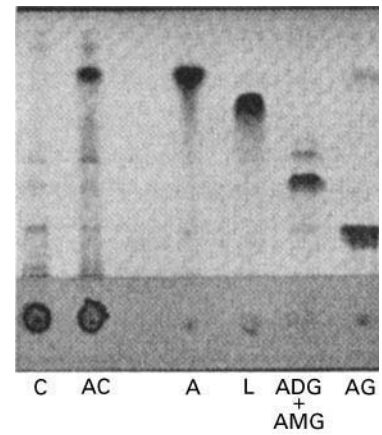

Figure 6 TLC chromatogram of camomile flavonoids. Key: C, dry extract of camomile ligulata flowers (CLF); AC, dry extract of autofermented CLF; A, apigenin; L, luteolin; ADG + AMG, mixture of apigenin-7-*O*-β-diacetylglucoside and apigenin-7-*O*-β-monoacetylglucoside; AG, apigenin-7-*O*-β-glucoside. Experimental details: silica gel 60 F254 HPTLC plates precoated with concentrating zone; eluent:benzol/ethylmethylketone/methanol (5 : 5.3 : 1.5); detection, $\lambda = 254$ nm. Yellow spots of separated flavonoids appear after spraying the plate with 96% sulfuric acid and heating briefly to 120°C. (Reproduced with permission from (1994) *Chromatography*, 39: 587–590.

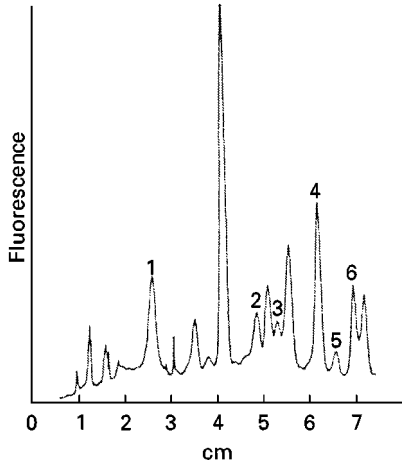

Figure 7 Densitogram of a camomile sample. Peaks: 1, apigenin-7-*O*-glucoside; 2, caffeic acid; 3, apigenin; 4, umbelliferone; 5, ferulic acid; 6, herniarin. (Reproduced from Menziani E, Tosi, B, Bonora A, Reschiglian, P and Lodi G (1990) *Journal of Chromatography*, 511: 396–401, with permission from Elsevier Science.)

determination is possible using appropriate instruments.

With the development of the automated multiple development (AMD) technique, planar chromatography may be applied successfully to the analysis of complex matrices. AMD-HPTLC gives the opportunity to carry out separation processes using a gradient development mode. An optimized AMD-HPTLC procedure has been applied to the separation of phenolic compounds (flavonoids, coumarin, phenylcarboxylic acids) of *Chamomilla recutita* flower extracts. **Figure 7** shows the densitogram obtained under optimized conditions: HPTLC plates and stepwise gradient development in an enclosed chamber. Fifteen steps were used, with drying times 6 min for the first four steps, then 4 min for the next 13 steps, and 10 min for the last step. As preconditioning, nitrogen was bubbled through water; the preconditioning time was 15 s for each step.

Cold-pressed citrus essential oils contain about 90–99% of volatile components, with a nonvolatile residue that ranges from approximately 1 to 10% in the different oils and consists, in large part, of many oxygen containing heterocyclic compounds, mainly the coumarins, psoralens and polymethoxylated flavones. The qualitative and quantitative composition of the nonvolatile residue characterizes the different citrus oils, and play an important role in identification, quality control and authenticity. The literature reports numerous TLC methods for the analysis or preparative isolation of oxygen heterocyclic compounds in citrus oils. An OPLC method has been developed to separate these components in seven citrus oils: sweet orange, bitter orange, mandarin, grapefruit, lemon, bergamot and Mexican lime. The OPLC separation is fast (10 min) and allows the differentiation of the various oils and the determination of possible contamination or sophistication of the oils.

In particular, many methods for the determination of bergapten (5-methoxypsoralen) have been reported, because of the problems linked with its phototoxicity. The *European Pharmacopoeia* (3rd edn) reports a TLC method to detect the presence of bergapten in bitter orange flower oil. Bergapten shows a greenish-yellow fluorescence at 365 nm. The presence of this psoralen may be an indication of the presence of bitter orange peel oil.

Conclusion

The examples described above show that both analytical and preparative, planar chromatography can be used successfully in a very wide range of applications to essential oils, balsams and resins. Thin-layer chromatography has the ability to separate mixtures of substances of similar structure and the flexibility of a variety of methods of detection. It also has the advantages of being cheap and easy to perform. As recently stated by J. Sherma in a review that appeared in *Analytical Chemistry* in 1996, 'fully instrumental planar chromatography, performed properly is the most economic, powerful, and accurate quantitative analytical method for mixtures of soluble substances of low vapour pressure'.

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